Bioactivity-Guided Fractionation of *Taxodium distichum*

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I. Abstract

Leishmaniasis is a parasitic disease with a high incidence of infection and, in some cases, the potential to be fatal. This disease is largely entrenched in poverty, making access to effective and inexpensive diagnoses, treatments, and disease control unattainable for countless infected individuals.[1] The need for affordable, more effective, and less toxic treatments has led us to the preparation and testing of plant extracts for screening against *L. donovani* parasites. Fractionation of the cones of a North American plant, found on The Ohio State University (OSU) campus and identified at the herbarium of The Ohio State University Museum of Biological Diversity as *Taxodium distichum* Rich. (Cupressaceae), commonly termed the bald cypress, has led to the observation of *in vitro* and *in vivo* activity against *L. donovani*. Previous studies have shown that abietane-type diterpenes, such as taxodione, taxodone, and taxodistines A and B are the major compounds present in the cones of *T. distichum*.[2-4] These compounds have previously been reported to have antitumor, antimicrobial, and antileishmanial activity, but unfortunately have also been found to be accompanied by serious undesired toxicity.[3-7] The purpose of this study was to isolate an antileishmanial compound or compounds from this plant source, which resulted in the purification and identification of taxoquinone and sugiol. Further fractionation is being conducted for the isolation and identification

of the particular chemical agent(s) responsible for the antileishmanial activity observed in the initial plant extract.

II. Introduction

Leishmaniasis is a significant public health concern in at least 88 countries worldwide, with approximately 12 million infected individuals.[8] Transmitted by an infected female sandfly vector, protozoan parasites of the genus *Leishmania* cause leishmaniasis via infiltration of tissue macrophages in host cells.[1] Leishmaniasis has varying clinical manifestations including cutaneous leishmaniasis (CL), as characterized by skin lesions, and visceral leishmaniasis (VL), affecting the internal organs. VL, caused by *L. donovani* in particular, is the more serious variety of the disease because it is highly fatal if left untreated.[1,8] The vast majority of leishmaniasis cases are concentrated in developing countries, with 90% of VL cases occurring in India, Bangladesh, Nepal, Sudan, and Brazil.[1] Due to the absence of affluence among the majority of infected individuals, they often lack access to effective diagnosis and treatment. Additionally, current treatments are known to be accompanied by adverse side effects, ranging from nausea to serious cardiotoxicity.[8] Isolation of endemic populations in developing countries has also stifled funding for and efforts toward the discovery of more effective treatments, which is becoming a growing concern as *Leishmania* species continue to develop increasing drug resistance to the most commonly employed

treatments in modern medicine, for example, miltefosine, amphotericin B, and pentavalent antimonial compounds such as pentostam.[1,8,9] Antimony is the first line of therapeutic action against leishmaniasis infections throughout the world, except in Bihar State in India, where the current cure rate in response to antimonial treatment has dropped to approximately 35%.[1] The substantial resistance to therapy in Bihar highlights the need for alternative treatments.

In addition to infecting approximately 1.5-2 million children and adults every year, leishmaniasis is associated with about 2.4 million disability-adjusted life years (caused by enduring physical impairments and/or scarring that alters the lifestyles of the affected individuals) and approximately 70,000 deaths annually.[1] Due to the high incidence of infection and the potential to be lethal, leishmaniasis has proven to be a pressing international health threat. There is a dense concentration of leishmaniasis cases in the Middle East, and the incidence of infection may be increasing there.[10] Mainly due to its impact on military operations, leishmaniasis awareness is increasing in industrialized countries that would otherwise have no significant contact with or interest in the disease.[11] United States military personnel have been affected during their service, with approximately 1300 cases of CL reported among individuals serving in Iraq and Afghanistan from 2003-2006.[11] As leishmaniasis continues to affect more lives of individuals from all over

the globe, the need for innovative treatments is becoming increasingly urgent.

III. Materials and Methods

The cones of *Taxodium distichum,* shown in Figure 1, were previously found and collected with the help and permission of grounds-keeping staff of The Ohio State University (OSU), Columbus, OH, at and around Mirror Lake. *T. distichum* is a deciduous conifer capable of attaining heights of approximately 40 meters native to the southeastern region of North America. This specimen was planted specifically as an ornamental tree on the OSU campus.[7,12]

Figure 1. *T. distichum* Cones

The detannified chloroform-soluble extract of the cones was obtained by solvent partitioning after methanol percolation, utilizing polarity to attain fractions desired in accordance with the partitioning scheme described by Kupchan and coworkers and later modified by Wall *et al.*, as

shown in Figure 2.[13] Partial detannification was necessary because polyphenolic compounds such as tannins have a well-known reactivity towards proteins, often producing false positive results in bioassays, particularly those involving enzymes.[13]

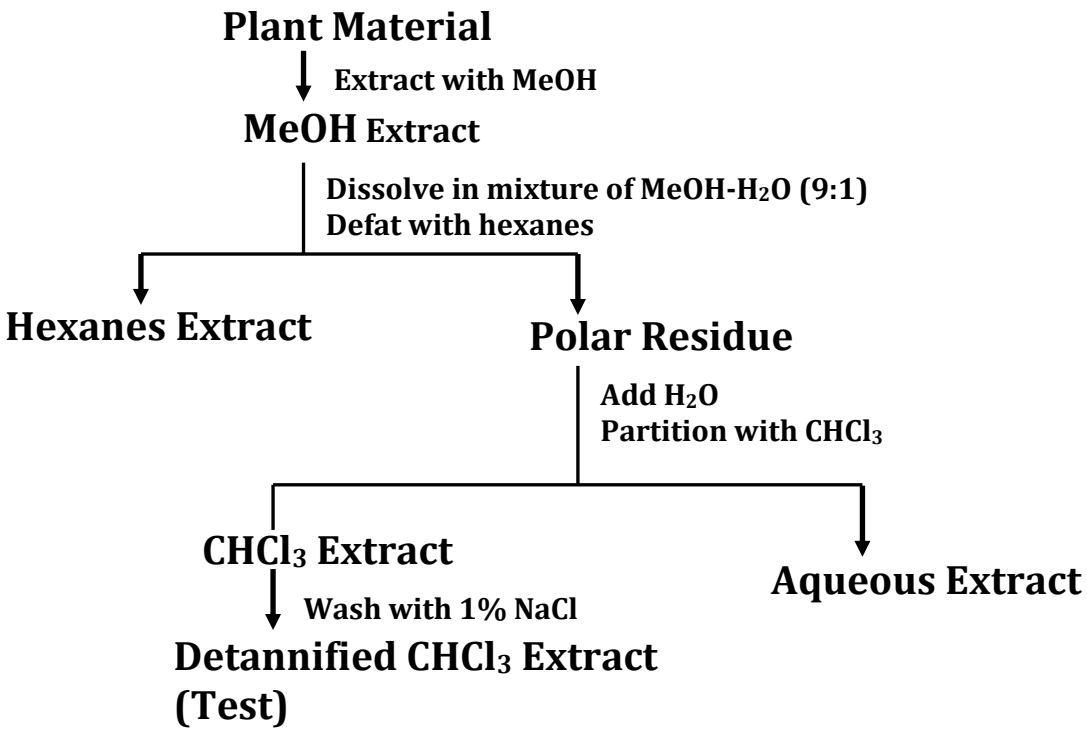


Figure 2. Partitioning Scheme According to Wall *et al*.[13]

The detannified chloroform-soluble extract of *T. distichum* cones (**TDCD3**) was tested against *L. donovani* promastigotes and found to be active, with IC_{50} = 2.1 µg/mL, supporting the proposed chromatographic fractionation to, ideally, purify and identify the observed leishmaniacidal agent(s). TDCD3 was applied to 40-63 µm normal-phase silica and separated with solvent systems of varying composition, based on polarity, by vacuum-liquid chromatography (VLC), as shown in Figure 3. VLC was

selected as the first means of separation in order to obtain an expedient and rapid set of subfractions. Due to the vacuum utilized, the separation may have been less efficient and precise than ideal, but this initial fractionation was useful for preliminary separation. Of the 14 fractions obtained, the second, termed TDCD3F2, displayed the only promising IC_{50} value after *in vitro* bioassay analysis against *L. donovani*, as shown in Table 1.

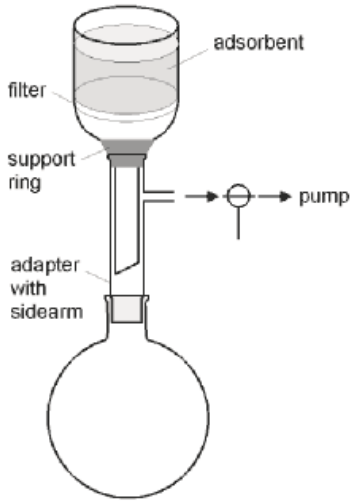


Figure 3. Vacuum-Liquid Chromatography Apparatus[14]

	Fractionation		IC_{50}
	Elution from Silica by VLC	*Sample Weight (g)*	*L. donovani promastigotes (µg/mL)*
TDCD3	*25 g loaded*	*0.113*	*2.1**
F2	*25% EtOAc*	*13.706*	*< 5*
F3	*25% EtOAc*	*2.361*	*> 10*
F4	*50% EtOAc*	*2.156*	*> 10*
F5	*50% EtOAc*	*0.843*	*> 50*
F6	*50% EtOAc*	*0.475*	*> 50*
F7	*75% EtOAc*	*0.657*	*> 50*
F8	*EtOAc*	*0.304*	*> 50*
F9	*EtOAc*	*0.105*	*< 100*
F10	*25% MeOH*	*1.284*	*> 50*
F11	*50% MeOH*	*0.355*	*> 25*
F12	*MeOH*	*0.094*	*< 100*
F13	*MeOH*	*0.053*	*> 50*
F14	*H2O*	*0.652*	*> 100*

Table 1. *In vitro* Bioassay Results of TDCD3 VLC Subfractions

Because TDCD3F2 exhibited the most potent inhibitory effect against *L. donovani*, it was selected for further fractionation. TDCD3F2 was also tested

in vivo in a murine model, and the results are shown in Figure 4. TDCD3F2 was tolerated in the mouse model and caused an observed dose-dependent decrease in liver and spleen parasite loads, further justifying further fractionation. This *in vivo* study was carried out by Dr. G. Gupta in the laboratory of Dr. A. R. Satoskar, Department of Pathology, Wexner Medical Center, OSU.

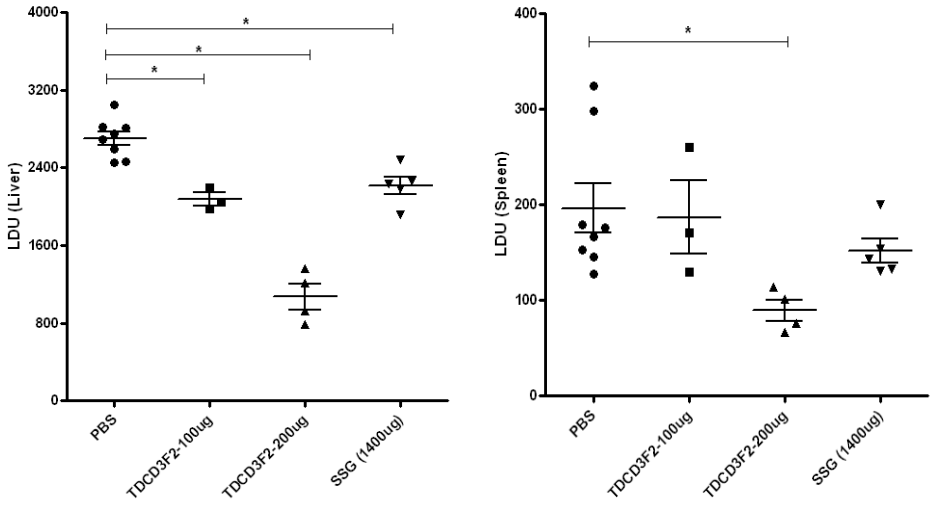


Figure 4. *In vivo* Results of TDCD3 Against *L. donovani* in a Murine Model. Liver and Spleen Parasite Loads Shown Above, Respectively.

Normal-phase 40-63 μm silica gel was utilized, and 7.1 g of TDCD3F2 were loaded onto a chromatographic column, and varying solvent systems used in elution comprised of 10:1 hexane-ethyl acetate, 5:2 hexane-ethyl acetate, and acetone, to elute the TDCD3F2 fraction through the silica matrix, relying only on gravity for this purpose. The eluates were collected in 104 separate sample tubes and analyzed by thin-layer chromatography (TLC) to determine those subfractions to

combine into master pooled fractions based on similarities in their polarities, as shown in Figure 5.

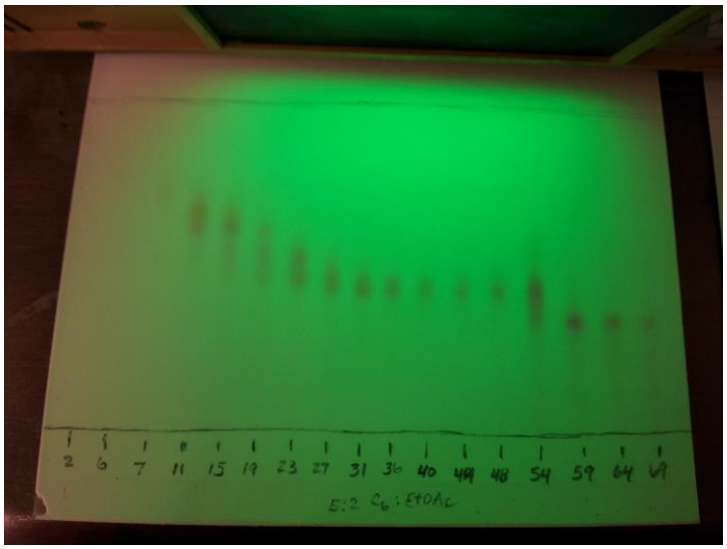


Figure 5. TLC Profiles of Selected TDCD3F2 Subfractions

Based on TLC results, the test tubes were combined into five pooled fractions, termed TDCD3F2.1 (comprised of fractions #1-19), TDCD3F2.2 (comprised of fractions #20-32), TDCD3F2.3 (comprised of fractions #33-51), TDCD3F2.4 (comprised of fractions #52-88), and TDCD3F2.5 (comprised of fractions #89-104). A light yellow precipitate formed in the TDCD3F2.1 fraction, which was separated from the mother liquor of TDCD3F2.1, resulting in TDCD3F2.1p (for precipitate) and TDCD3F2.1m (for mother liquor). Moving forward, the TDCD3F2.1p fraction was not considered to be abundant enough to work up, but TDCD3F2.1m was. These five master fractions were tested *in vitro* against *L. donovani* promastigotes, with the results shown in Table 2.

In vitro Biological Activity Evaluation

Transgenic DsRed2 *Leishmania donovani* promastigotes (expressing red fluorescent protein) were grown in Medium 199 substituted with 10% FBS and 1% penicillin and streptomycin. They were passaged infecting Stat-4 KO mice (immunocompromised).

Using a previously published procedure,[15] *L. donovani* promastigotes as described above were placed into 24-well culture plates ($1x10^6$ cells/mL/well) and exposed to different concentrations of drugs or test samples in duplicate for 72 hours at 23°C. Untreated promastigotes and those treated with 1 mg/mL saponin for 1 hour were kept as negative and positive controls, respectively, for the experiment. Miltefosine was used as a standard antileishmanial drug in this study. The drug-induced *Leishmania* killing was quantified by flow cytometry using a Fluorescence-Activated Cell Sorter (FACS). IC_{50} values (drug concentrations that resulted in 50% inhibition of parasite growth) were determined by GraphPad Prism® software using non-linear regression (Log inhibitor vs. Response on a variable slope).

Identity	IC_{50} (µg/mL)
Miltefosine	1.8
TDCD3F2.1m	0.8
TDCD3F2.2	1.0
TDCD3F2.3	1.9
TDCD3F2.4	12.7
TDCD3F2.5	>25

Table 2. *In vitro* Bioassay Results for TDCD3F2 Subfractions

TDCD3F2.1m, TDCD3F2.2, and TDCD3F2.3 exhibited strong inhibitory activity against *L. donovani*, with TDCD3F2.1m and TDCD3F2.2 having IC_{50} values lower than that of the miltefosine standard. TLC evaluation of these three potent fractions revealed similarities in their constituents, implying that they all could have contained the same active analyte(s). Since TDCD3F2.1m and TDCD3F2.2 were more potent than miltefosine and displayed the most similarities based on TLC analysis, shown in Figure 6, these two fractions were combined to produce TDCD3F2.1m2 in order to concentrate the potentially leishmaniacidal agent(s) and attempt isolation by further chromatographic separation and purification. Trace similarities were present in TDCD3F2.3, which could explain its biological activity, but there were more striking differences in its content according to TLC. In the interest of attempting to isolate the active agent, which seemed to be more concentrated in TDCD3F2.1m and TDCD3F2.2, TDCD3F2.3 was not combined with TDCD3F2.1m2 because its dissimilarities might have complicated the precise separation upon further work up.

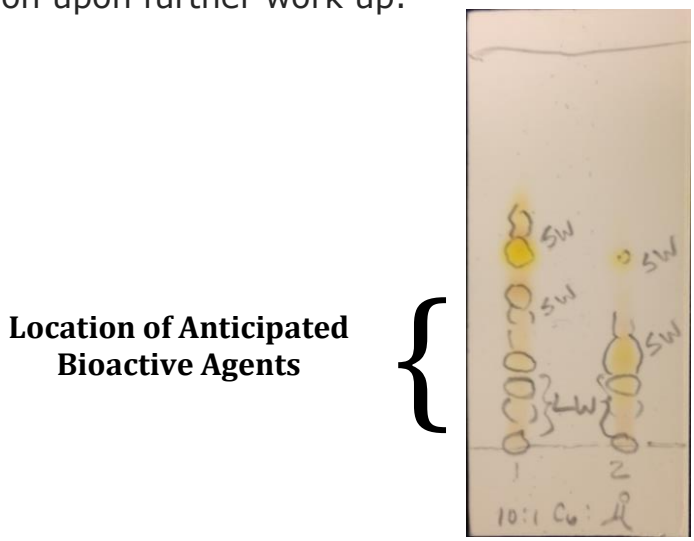


Figure 6. TLC Profiles of Bioactive Fractions TDCD3F2.1m (1) and TDCD3F2.2 (2)

Approximately 4.2 g of TDCD3F2.1m2 were added to a glass column loaded with 40-63 μm normal-phase silica gel. Varying solvent systems comprised of 20:1 hexane-acetone, 10:1 hexane-acetone, and 5:2 hexane-acetone were employed and the eluate(s) were collected to obtain a total of 80 subfractions in separate test tubes. Fractions #12-16 developed dark orange/brown crystals, shown in Figure 7, and fractions #42-47 developed light orange crystals without intervention. The crystals resulting from #12-16 were combined and purified by recrystallization in hexane/acetone to yield approximately 10 mg of bright orange crystals, shown in Figure 8, which were termed compound **1**.

Figure 7. Crude Crystals of 1

Figure 8. Purified Crystals of 1

Compound **1** was identified as taxoquinone by comparison of 400 MHz ^{1}H- and 100 MHz ^{13}C-NMR spectroscopic data with previously published values.[16,17] The crystals resulting from #42-47 were combined and purified by high-pressure liquid chromatography (HPLC) with a 77% methanol/23% water solvent method, yielding approximately 15 mg of white crystalline

solid after drying, termed compound **2**. Compound **2** was identified as sugiol by comparison of NMR data with published values.[18,19] The structure and NMR spectra of compound **1** are shown in Figures 9 and 10, while the structure and NMR spectra of compound **2** are shown in Figures 11 and 12.

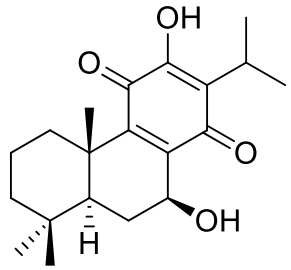
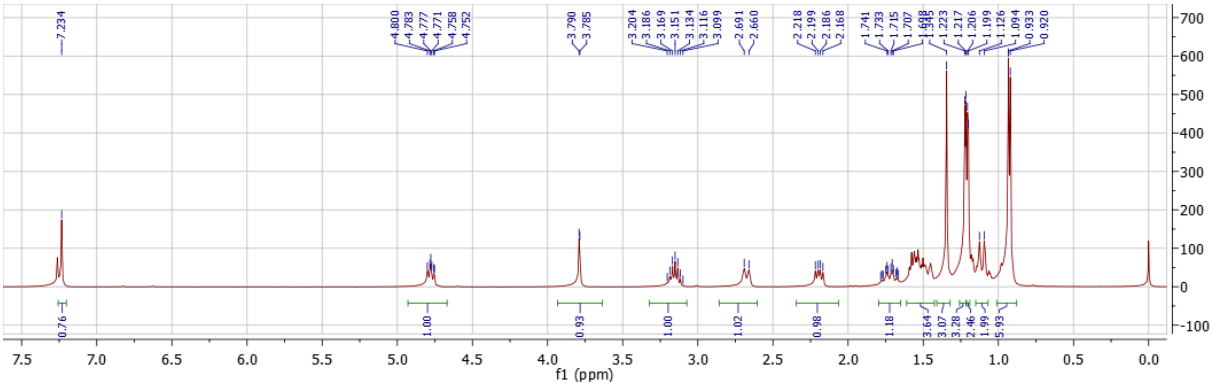


Figure 9. Taxoquinone Structure (left) and ^{1}H-NMR Spectrum (below)



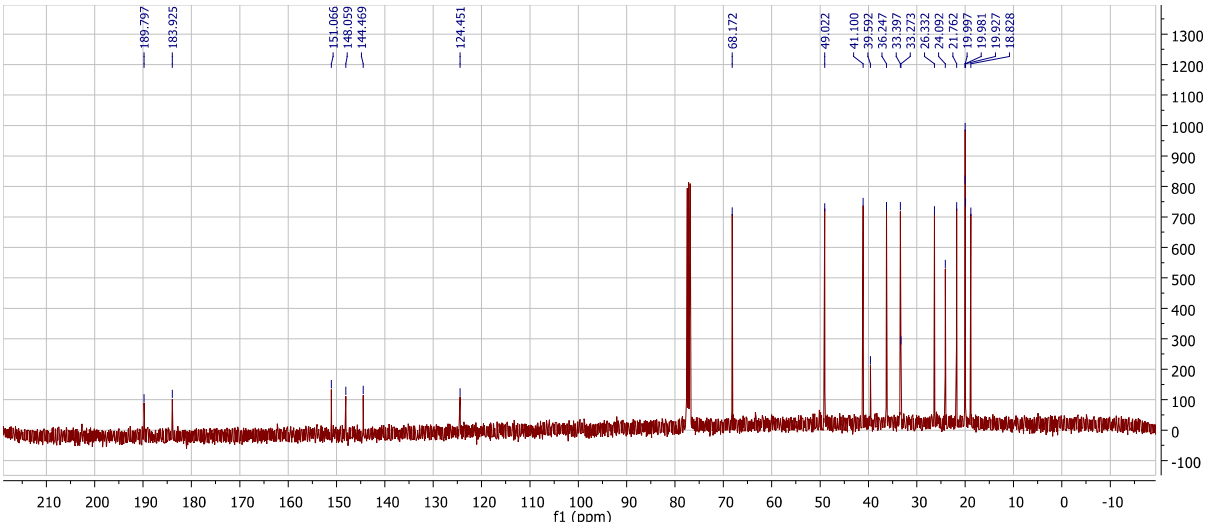


Figure 10. Taxoquinone ^{13}C-NMR Spectrum (100 MHz, $CDCl_3$)

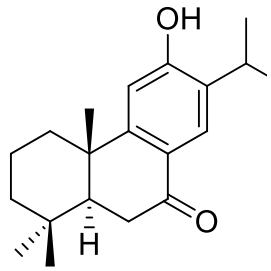
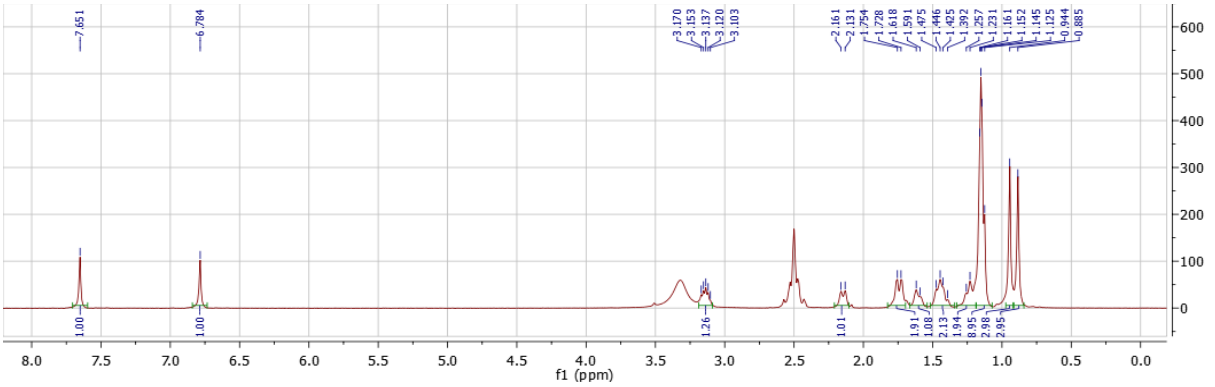


Figure 11. Sugiol Structure (left) and ^{1}H-NMR Spectrum (below)



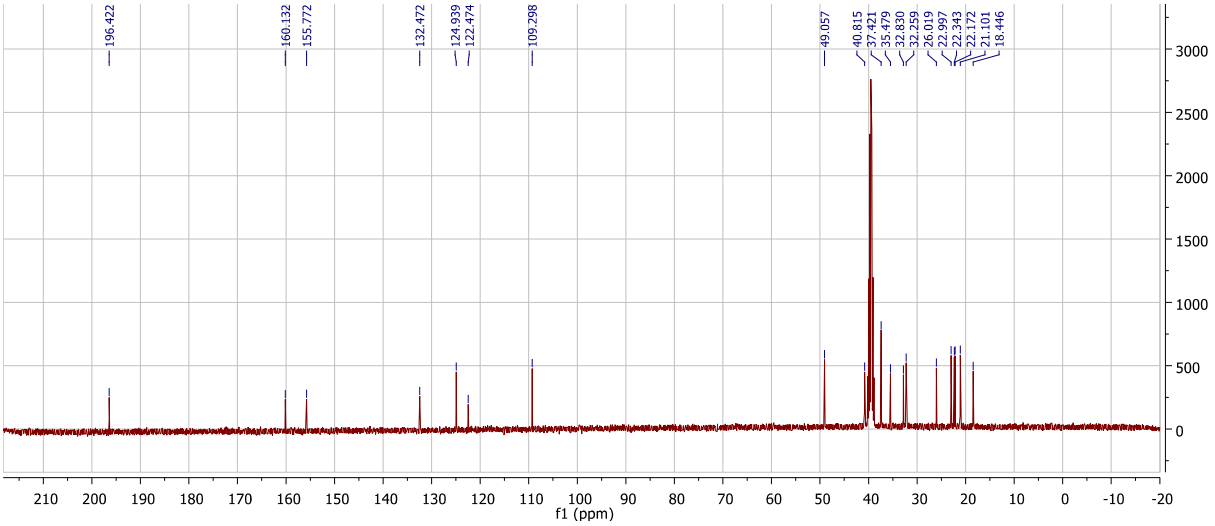


Figure 12. Sugiol ^{13}C-NMR Spectrum (100 MHz, DMSO-d_6)

The 80 total fractions were analyzed by TLC and combined into nine master fractions: TDCD3F2.1m2-A (comprised of fractions #1-6), TDCD3F2.1m2-B (comprised of fractions #7-11), TDCD3F2.1m2-C (comprised of #12-19), TDCD3F2.1m2-D (comprised of fractions #20-24),

TDCD3F2.1m2-E (comprised of fractions #25-33), TDCD3F2.1m2-F (comprised of #34-47), TDCD3F2.1m2-G (comprised of fractions #48-58), TDCD3F2.1m2-H (comprised of fractions #59-70), and TDCD3F2.1m2-I (comprised of fractions #71-80). Due to the limited availability of viable parasites necessary for biological activity evaluation, not all fractions could be tested in the bioassay. Compounds **1** and **2**, and fractions C-G were tested in bioassay against *L. donovani* promastigotes utilizing the same procedure used for the previous set of fractions, set up shown in Figure 12. Results are shown in Table 3 and Figure 13.

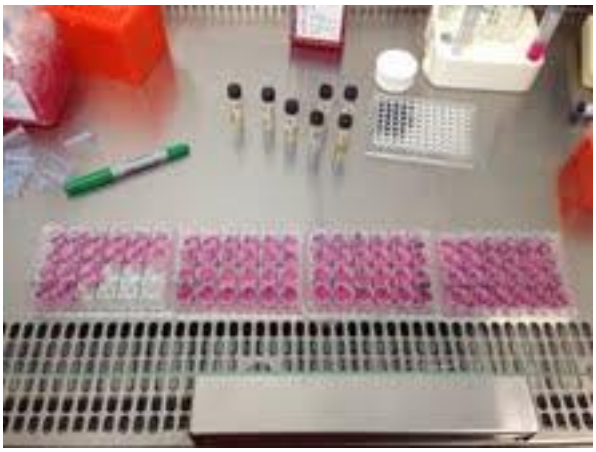


Figure 12. *In vitro L. donovani* Promastigote Bioassay Set Up

Identity	IC_{50} (µg/mL)
Miltefosine	1.8
Compound **1**	>25
Compound **2**	10.4
TDCD3F2.1m2-C	2.1
TDCD3F2.1m2-D	0.5
TDCD3F2.1m2-E	0.5
TDCD3F2.1m2-F	1.6
TDCD3F2.1m2-G	1.9

Table 3. *In vitro* Bioassay Results of TDCD3F2.1m2 Subfractions against *L. donovani* Promastigotes

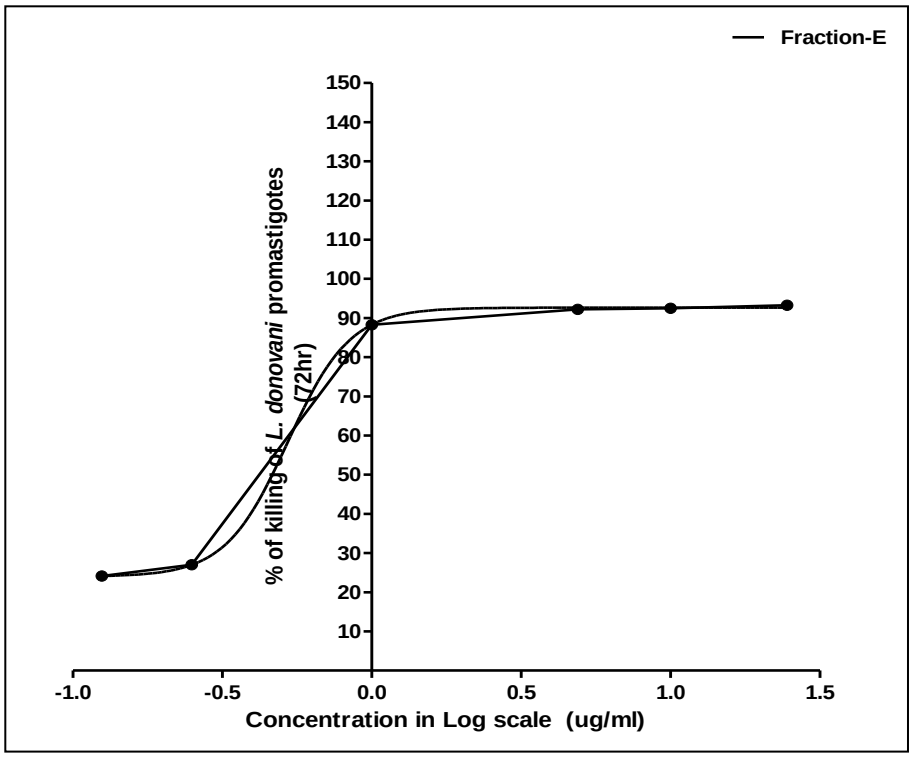


Figure 13. Potent Inhibition of *L. donovani* by TDCD3F2.1m2-E

IV. Conclusions

The screening of *Taxodium distichum* cone subfractions has led to the identification of selected subfractions as samples possessing *in vivo* and *in vitro* leishmaniacidal utility, especially TDCD3F2.1m2-D and TDCD3F2.1m2-E, which were significantly more potent than the reference drug miltefosine *in vitro*. The results also show that compounds **1** and **2** are inactive and weakly active against *L. donovani* promastigotes, respectively. As drug resistance becomes more prevalent among *Leishmania* species, the need for alternatives to current treatments is more pressing than ever before. The identification of lead compounds is crucial to the development of new therapies for pressing health issues such as leishmaniasis. With IC_{50} values

lower than those of some other treatments currently being employed, the *T. distichum* constituent(s) in question that produce the activity of the tested active fractions could qualify as lead compound(s) for drug development. However, further purification and more *in vivo* toxicology evaluation of these compounds must be completed before this could be tested in a human system. Also, tentative results from *in vitro* intracellular amastigote analysis exhibited marked decreases in observed potency against *L. donovani*, implying that there could be complications interacting with the amastigote developmental stage and/or with the ability to efficiently cross biological membranes to infiltrate infected cells. Moving forward, more precise fractionation by HPLC of the subfractions is necessary to isolate and later identify the agent(s) responsible for the antileishmanial character of those active subfractions. Natural products hold incalculable potential to counteract disease, which is why their investigation should be continued in an effort to identify other promising leads with potential to be developed into therapeutic agents, aiding in the global fight against leishmaniasis and other infectious diseases.

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